INHIBITION OF BLOOD DIGESTION AND OOCYTE GROWTH IN AEDES AEGYPTI BY 5-FLUOROURACIL

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5-Fluorouracil (FU) inhibits oviposition in house flies, screw-worm flies and fruit flies (Smith *et al.*, 1964). This compound sterilizes only females (Crystal, 1963) and its sterilizing effect is temporary (Painter and Kilgore, 1964). Only the final effect of FU-treatment, *i.e.*, the inhibition of oviposition, has been observed in these studies. It is not known at what stage egg maturation was arrested, and whether any yolk was formed.

The morphological and physiological changes which occur in the ovaries and also in extraovarian tissues during egg maturation in *Aedes aegypti* have been extensively studied, and summarized in Christophers' (1960) and Clements' (1963) monographs. In this insect egg maturation is initiated by a blood meal; this fact makes it possible to recognize the sequence of events leading to egg formation, and their timing in relation to the blood meal.

Before the blood meal the ovaries are in a "resting stage." Shortly after gorging, a hormonal factor is released which initiates oocyte growth. Four hours after the blood meal, yolk deposition is already evident in the oocyte (Laurence and Roshdy, 1963). Yolk protein uptake by the oocyte is most active seven hours after the blood meal; yolk synthesis and storage are essentially completed 25 hours after the meal (Roth and Porter, 1964).

Very little proteolytic activity can be detected in the midgut of *Aedes aegypti* before the blood meal. Midgut protease activity rapidly rises in the first few hours and reaches its peak 18–24 hours after engorgement (Fisk and Shambaugh, 1952). Two days after a blood meal (at 28° C.) blood digestion is completed and the ovaries contain mature eggs.

This paper describes the effect of FU on the rate of oocyte growth and blood digestion in *Aedes aegypti* females.

Materials and Methods

Aedes aegypti mosquitoes ("Ness-Ziona" strain, kept at this laboratory since 1950) were reared under standard laboratory conditions at 28° C. FU (Hoffmann La-Roche) was given in 5% sucrose for two days, or in sheep blood (containing 0.25% sodium citrate) through Silver-light membranes (Galun *et al.*, 1963). Adult mosquitoes, before and after treatment, were kept on 5% sucrose at 28° C.

In oviposition experiments single engorged females were confined in gauze-covered 170-ml. glass jars, containing a small aluminum cup filled with water and lined with a strip of filter paper (these females were fed on honey). The day of the oviposition was noted, and the eggs were kept for a further three days in moisture at 28° C. and then dried. The number of eggs and the percentage of

hatching were determined for each batch. Hatching was stimulated by lowering the oxygen tension in the water containing the eggs (Clements, 1963). Each batch of eggs was immersed in tap water in a 10-ml. beaker; the beakers were placed in a desiccator, and the pressure was reduced to 70 mm. Hg. for 10–20 minutes to remove the air from the water.

At daily intervals after the blood meal the females were dissected in 0.85% NaCl and the size of the ovarian follicles was measured under a binocular microscope. The ovarian development was divided into eight stages, based on Christophers' classification (1960) as modified by Clements (1963). Stage 1 is the resting stage, corresponding to Clements' stage IIa, stage 2 corresponds to IIb. We have subdivided stage III into three stages (3–5), according to the amount of yolk formed. Our stages 6–8 correspond to Clements' (1963) stages IVa, IVb and V, respectively. The division into eight stages enabled us to calculate the average stage of the ovaries at various intervals after a blood meal.

Stage 1. No yolk visible under low-power (100 ×) magnification (resting stage).

Stage 2. Yolk granules visible in the oocyte under a binocular (ovaries have passed the resting stage).

Stage 3. The oocyte (20–50 microns in length) occupies $\frac{1}{4}$–$\frac{1}{3}$ of the follicle.

Stage 4. The oocyte (51–100 microns in length) occupies $\frac{2}{5}$–$\frac{3}{4}$ of the follicle.

Stage 5. The oocyte (101–150 microns in length) occupies about $\frac{4}{5}$ of the follicle.

Stage 6. The oocyte (151–250 microns in length) occupies about $\frac{9}{10}$ of the follicle.

Stage 7. The follicles (251–350 microns in length) assume the shape of the mature eggs.

Stage 8. The ovary contains mature eggs with visible chorionic structures.

The stages of blood digestion (presented schematically by Stohler, 1957) can be easily observed under low magnification. The color of the ingested blood is bright red in freshly engorged females. Digestion begins at the periphery within the gut's lumen, and can be recognized by the brownish color around the periphery several hours after the meal (Stohler's stages A and B). Blood digestion proceeds from the periphery inwards. About one day after engorging, the blood mass is brown except for the center (Stage C). When the color of the blood mass is brown throughout, digestion is completed and elimination begins. The size of the blood mass decreases and the midgut walls become again folded (stage D).

Blood-fed females were dissected in 0.85% NaCl at daily intervals after the blood meal, and 12–30 midguts from each group were kept in a small glass vial at −18° C. until needed (no decrease of protease activity was observed after two months' storage at −18° C.). The protease activity was tested by a modified procedure of Tomarelli *et al.* (1949), using azoalbumin as substrate. The midguts were homogenized in a glass homogenizer in cold 0.1 *M* sodium phosphate buffer, pH 7.8, containing 1:20,000 thiomersalate (0.1 ml. buffer for each gut). Azoalbumin was dissolved in the same buffer, 25 mg. per ml. The incubation mixture consisted of 0.3 ml. 0.1 *M* buffer, pH 7.8 (which was shown to be optimal by Fisk, 1950), 0.4 ml. substrate solution (containing 10 mg. azoalbumin) and 0.3 ml. midgut

homogenate (equivalent to three midguts). All experiments were run in duplicate. The tubes were incubated for 30 minutes at 39° ±0.1° C. The reaction was terminated by the addition of 3.0 ml. 6% trichloroacetic acid. The precipitated protein was separated by centrifuging and filtering. Three ml. of the supernatant from each tube were mixed with 3.0 ml. 0.5 N NaOH, and optical densities were determined at 440 millimicrons. Protease activity was measured as mg. azoalbumin digested by three midguts in 30 minutes.

Results

Effect of feeding FU in the blood meal on oviposition

Six-day-old mated females were given FU in sheep blood through membranes. The FU in the blood meal did not interfere with normal feeding, but all the engorged females died within one day when the final concentration of FU was 0.5%. With 0.25% FU 30% mortality occurred; lower concentrations did not affect the viability. The minimal dose which inhibited oviposition was 0.025% FU (Table I).

Table I

Effect of feeding FU in the blood meal on oviposition

% FU	Oviposition after 1st blood meal (Sheep blood mixed with 0.85% NaCl)			Oviposition after 2nd blood meal (on rat)		
	No. of females ovipositing	Eggs per ovipositing female*	% inhibition of oviposition**	No. of females ovipositing	Eggs per ovipositing female	% inhibition of oviposition
none	36/47	51.2	—	54/55	101.0	—
0.005	7/14	45.7	41.7			
0.010	0/18	9	98.7			
0.025	0/20	—	100			
0.05	0/20		100	24/43	32.2	81.8
0.25	0/19		100	4/28	35.0	95.0

* The percentage of hatching was over 80% in all batches of eggs.

** $\frac{\text{Eggs per female in control—eggs per female in treated}}{\text{Eggs per female in control}} \times 100.$

Since the amount of blood taken up by a fully engorged female is about 2 mg., the LD_{50} is between 5 and 10 μg. FU, and the dose which causes 50% inhibition of oviposition between 0.1 and 0.2 μg. FU per female. FU-treatment did not affect the viability of the eggs laid; 83–89% of the eggs laid by the treated females hatched, as compared with 91% in the controls.

The effect of FU on oviposition was not permanent. More than half of the females which ingested 0.05% (two times the minimal dose which inhibited oviposition) and over 10% of those which took 0.25% (10 times the minimal dose inhibiting oviposition) in their first blood meal, oviposited viable eggs after a second blood meal (on a rat), which was given one week after the FU-treatment (Table I).

The blood meal, consisting of one part of 0.85% NaCl to three parts of sheep blood, was nutritionally suboptimal. About a quarter of the engorged controls did not lay any eggs, and the average number of eggs laid was only about half of

that following a blood meal on a rat. In order to study the effect of FU under conditions of optimal nutrition, it was more convenient to treat the females first and then feed them on a rat.

Effect of feeding FU in sugar solution on oviposition

One-day-old mosquitoes were fed FU in 5% sucrose for two days, kept on sugar solution for two more days, and then offered a rat. The average weight of the blood meal taken by the females treated with 0.05% FU was not less than that taken by the controls (2.3 mg. and 2.16 mg., respectively). Egg production in mosquitoes treated with 0.05% FU two days before their blood meal was greatly inhibited (see Table II. The results with 0.05% FU were pooled from 5 experiments, each consisting of 10–12 engorged females; in two experiments no oviposition occurred).

TABLE II

Effect of feeding FU in sugar solution on oviposition
(FU in 5% sucrose for two days to one-day-old mosquitoes, blood meals on rat)

Time of first blood meal	% FU	Oviposition after 1st blood meal			Oviposition after 2nd blood meal		
		No. of females ovipositing	Eggs per ovipositing female*	% inhibition of oviposition**	No. of females ovipositing	Eggs per ovipositing female	% inhibition of oviposition
Rat two days after removal of FU	none	49/50	93.1	—	23/24	93.7	—
	0.0125	18/24	80.7	33.6	13/13	87.6	2.4
	0.025	15/24	63.6	57.3	14/14	75.9	15.4
	0.05	5/54	54.2	94.5	16/28	56.5	64.0
Rat 14 days after FU	none	14/14	102.4	—			
	0.05	4/9	11.5	94.7			

* The percentage of hatching was over 80% in all batches of eggs.

** $\frac{\text{Eggs per female in control—eggs per female in treated}}{\text{Eggs per female in control}} \times 100.$

When the interval between FU-treatment (0.05%) and the blood meal was prolonged from two to 14 days, a greater percentage of females was able to lay eggs, though there was little recovery in terms of the average number of eggs per female. The recovery of the females treated with 0.05% FU was more obvious after the second blood meal (Table II).

The eggs laid by the females treated with FU in sugar solution also hatched normally (80–88%). Although FU in sugar solution was given to both sexes, it evidently affected only the oviposition, but had no effect on the viability of the eggs.

Table II gives only the number of eggs per female after the first and the second blood meal. However, FU also affected the time of oviposition. The controls that fed on rats started ovipositing two days after feeding, and all of them completed oviposition within three days after the blood meal. Females that were treated with 0.0125% and 0.025% FU started ovipositing 4–5 days after feeding on blood; those treated with 0.05% FU oviposited 5–7 days after their first blood meal. After the second blood meal oviposition was also later than in the controls, but only in the

group treated with 0.05% FU. Dissection of the mosquitoes showed that the delay in oviposition in the treated females was due to slower maturation of the oocytes.

Effect of FU on the rate of oocyte growth

The ovaries of gravid females were examined daily after feeding on a rat, until oviposition occurred, and the stage of oocyte development was determined. (Twenty to 80 females were dissected daily in each group.) The stages of ovarian development were divided into 8 stages as described in Materials and Methods. The average stage of the ovaries on each day after the blood meal, and the percentage of females containing mature eggs, are given in Figure 1.

The rate of oocyte growth and the percentage of females developing mature eggs was proportional to the amount of FU given and the time interval between FU-

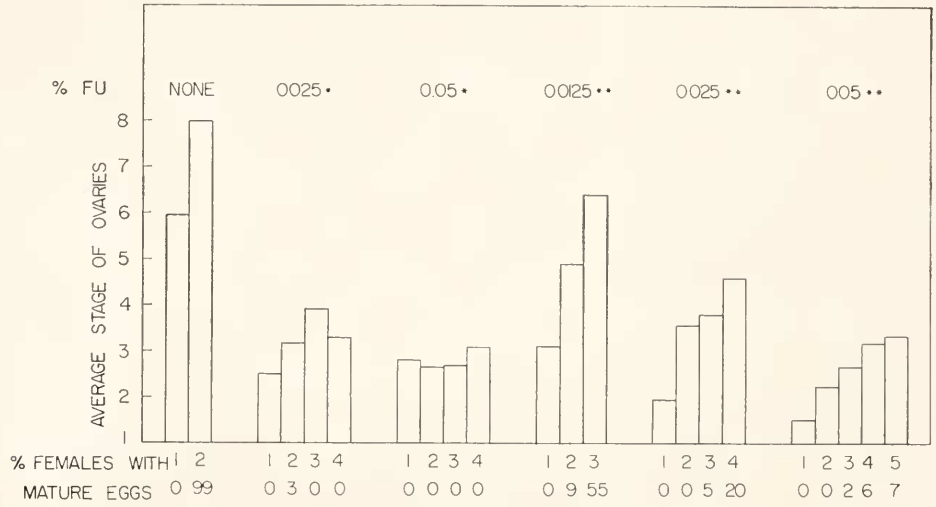

FIGURE 1. Effect of FU on the rate of ovarian development (FU in 5% sucrose for two days, blood meals on rats). The numbers below the columns indicate the day after the blood meal. *Blood meal immediately after FU. **Blood meal two days after FU.

treatment and the blood meal. When five-day-old mosquitoes were fed 0.05% FU for two days immediately before their blood meal oocyte growth was completely inhibited beyond stage 5. The ovaries of most of the females remained in stages 2 and 3, and only 2% of all dissected females had reached stage 5. When the first blood meal was given two days after 0.05% FU, a few females were able to develop mature eggs (Fig. 1).

The ovaries of the treated females which were in stages 2–4 could not be distinguished from normal ovaries in a comparable stage of development. However, some of the ovaries of treated mosquitoes, which developed beyond stage 4, showed irregular development (irrespective of the concentration of FU). Normally all the growing follicles in mosquito ovaries are of uniform size. In some of the FU-treated females the rate of growth of individual follicles varied. A small number of follicles (often only one or a few per ovary) had reached stages 6–8, whereas

the remainder was of a size typical of stage 4 ovaries. This asynchronous development affected both ovaries. The stage of ovarian development assigned to asynchronous ovaries was that of the largest follicle present (for the computation of the averages in Figure 1). Ovaries were considered as stage 8 even when only a few follicles developed mature eggs.

A second blood meal was offered 8 days after the first (10 days after removal of FU). The rate of egg maturation (and also the number of eggs produced, see Table II) in the females treated with the lower concentrations of FU was almost equal to that of the controls. Two days after the blood meal 96.2% and 87.5% of the females treated with 0.0125% and 0.025% FU, respectively, contained mature eggs. In females treated with 0.05% maturation of eggs was slower. Only 33% contained mature eggs two days after the second blood meal; the ovaries of the rest were in stages 4–6.

Effect of FU on the rate of blood digestion

In untreated females blood digestion at 28° C. is completed about 36 hours after feeding on a rat, and defecation begins. Forty-two hours after feeding 66% of the females had empty midguts; 33% contained small blood residues (a quarter of the original amount). Of 152 females dissected 48 hours after the blood meal, 23% contained blood residues in the midgut or in the hindgut (Fig. 2). No traces of the blood meal were evident in untreated females 54 hours after engorging.

The females treated with FU immediately before the blood meal, or two days before the blood meal, retained the blood in their midguts much longer than the controls (Fig. 2). The delay in defecation was proportional to the amount of FU given.

The time of 50% digestion is the time in hours after a blood meal, when 50% of the females had completely emptied their guts (Fig. 2). The point of 50% digestion time was obtained by interpolation between the two adjacent days (when less or more than 50% of the midguts were empty) on log probability paper. The results in Figure 2 indicate that the period of blood digestion in females treated with 0.05% FU is more than twice that in the controls.

In our oviposition experiments we measured the number of eggs per blood meal. If the mosquitoes had free access to blood meals, oviposition could be measured per unit of time. The controls could thus have taken more blood meals and accomplished more gonotrophic cycles than the FU-treated females. The inhibition of oviposition by the lower concentrations of FU would be more pronounced under these conditions than in our experiments (Table II).

All the females whose ovaries reached stage 8 had completed blood digestion by that time. The females whose ovaries remained undeveloped retained the blood longer, but eventually emptied their midguts without developing mature eggs.

The rate of blood digestion returned to normal after the second blood meal in the females treated with 0.025% and 0.025% FU (in these groups maturation of eggs also proceeded at a normal rate). Recovery after the second blood meal was slower in the females treated with 0.05%.

The females whose ovaries had reached stage 8 two days after the second blood meal had also completed blood digestion by that time (see above). The midguts of 66% of the females whose ovaries had not developed beyond stage 6 were still

full two days after feeding (*i.e.*, blood digestion and ovarian development were in the stage reached by the controls one day earlier).

The delay in blood digestion in the FU-treated females was evident even before defecation started. The fact that blood digestion was greatly retarded in the treated females could be observed by the color of the blood mass one day after feeding. The whole blood mass in the untreated females was brown, whereas the blood mass in the females treated with 0.05% was bright red except for a thin brownish layer at the periphery. Blood digestion (as estimated by the color of the blood mass one day after feeding) had proceeded further in the females treated with 0.0125% and 0.025% FU, but did not reach the stage of the controls. Since

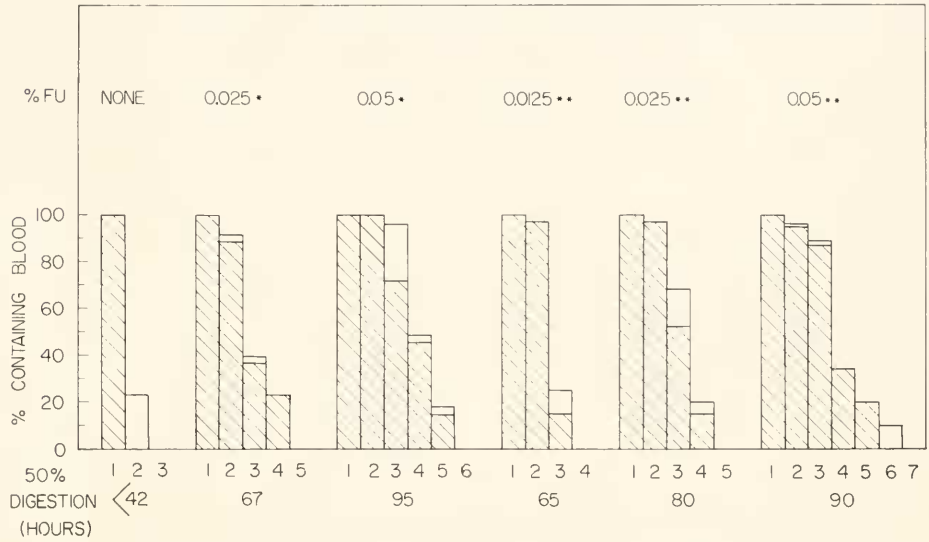

FIGURE 2. Effect of FU on the rate of blood digestion (FU in 5% sucrose for two days, blood meals on rats). The numbers below the columns indicate the day after the blood meal. Shaded columns indicate full midguts; empty columns blood residues. * Blood meal immediately after FU. ** Blood meal two days after FU.

the changes in the color of the blood mass indicate the extent of proteolytic activity, the midgut protease of the FU-treated females was examined *in vitro*.

Effect of FU on midgut protease

The protease activity was measured as mg. azoalbumin released by a homogenate of three midguts (including the content of the lumen) after 30 minutes' incubation at 39° C. The reaction proceeded linearly up to about 30 minutes; after that the rate of the reaction decreased gradually. There was little proteolytic activity after three hours. No protease activity could be detected by our method in untreated midguts dissected immediately after engorging. The average protease activity 4–5 hours after feeding was about 0.15 mg. per three midguts. Protease activity of untreated midguts reached its peak 18–24 hours after feeding. At

that time the average protease activity was 0.874 ± 0.135 (standard deviation) mg. azoalbumin released by three midguts in 30 minutes at 39° C. (13 experiments). The protease activity of untreated midguts was low 48 hours after blood meal (0.028 mg., average from four experiments), but only 25% of the midguts contained visible blood residues; the rest were empty.

The protease activity of homogenates prepared from a mixture of empty and blood-containing midguts is due to the blood-containing midguts. No protease could be detected by our method in midguts which have recently been emptied (42 hours after feeding). The average protease activity of midguts which still con-

TABLE III

Midgut protease activity in FU-treated females. (FU in 5% sucrose for two days)

Treatment	% FU	Day after blood meal	% with blood-filled midguts	Average protease activity*
FU given to 5-day-old mosquitoes. Blood meal immediately after removal of FU	none	1	100	0.77
	0.025	1	100	0.15
		2	89	0.34
		3	36	0.27
		4	23	0.21
	0.05	1	100	0.15
		2	100	0.22
		3	72	0.12
		4	45.5	0.15
FU given to one-day-old mosquitoes. Blood meal two days after removal of FU	none	1	100	0.86
	0.0125	1	100	0.33
		2	100	0.61
	0.025	1	100	0.20
		2	95	0.43
		3	52.5	0.30
	0.05	1	100	0.19
		2	95	0.26
		3	75	0.41

* Mg. azoalbumin per three midguts, incubation 30 minutes at 39° C. Only blood-filled midguts, 12–30 for each test, were taken.

tained visible traces of the blood meal 42–46 hours after feeding was 0.11 mg. azoalbumin per three midguts.

Fisk and Shambaugh (1952) found a small amount of "residual" protease in unfed and sugar-fed mosquitoes (after 5 hours of incubation). This amount was too small to be detected by our technique. Our results regarding the amount of protease at various times after a blood meal in untreated females are similar to those obtained by Fisk and Shambaugh, if adjusted to the differences in the time of incubation. In their experiments considerable protease activity was found 48

hours after feeding, but they kept the females at a lower temperature. Blood digestion in *Aedes aegypti* is known to be slower at lower temperatures (Stohler, 1957).

The midgut proteolytic activity of FU-treated females is given in Table III. Only midguts that were fully distended with blood were taken for the protease assay (in order to make sure that we measured the protease activity before the peak, or near to it, and not the residual protease after digestion had been completed). The percentage of females containing blood-filled midguts on each day after feeding is given in Table III. The protease activity of the controls two days after feeding is not included, since none of the controls had blood-filled midguts by that time.

The inhibition of the protease activity in the FU-treated females was most obvious 24 hours after blood meal, when the protease activity in the controls was at its peak. In the treated females protease activity was higher two days after feeding than on the preceding day. Though the peak protease activity in the treated females occurred 1–2 days later than in the controls, the amount of pro-

TABLE IV

Recovery of midgut proteolytic activity after FU-treatment (0.05% FU in 5% sucrose given to one-day-old mosquitoes for two days. Blood meals on rat)

Time of blood meal	Day after blood meal	Average proteolytic activity*	
		Treated	Controls
First meal two days after FU	1	0.14	1.07
	2	0.23	—
	3	0.23	—
First meal 7 days after FU	1	0.10	0.85
	2	0.33	—
First meal 14 days after FU	1	0.36	0.84
Second meal 10 days after FU	1	0.64	0.84

* Mg. azoalbumin per three blood-filled guts, incubation 30 minutes at 39° C.; 12–20 midguts were taken for each test.

tease in FU-treated females never reached the peak values of the controls. Protease inhibition was higher when the blood meal was given immediately after the FU treatment, and it was proportional to the amount of FU (Table III).

In order to test whether inhibition of protease by FU was caused by the direct inactivation of the enzyme, the effect of FU was tested *in vitro*. One and 2 mg. of FU, dissolved in 0.1 *M* sodium phosphate buffer, pH 7.8, was added to homogenates of midguts from untreated females (dissected 24 hours after feeding, *i.e.*, at the peak protease activity). FU did not inhibit protease activity *in vitro*.

The inhibition of protease activity by FU persisted for many days after FU-treatment (Table IV). Protease activity was tested only on the days when all midguts were still filled with blood; the data thus represent the average of the whole population. Though protease activity one day after feeding increases when

the blood meal is postponed from two to 7 and 14 days after FU treatment, it does not reach the level of the controls. Inhibition of protease activity was also evident after the second blood meal (76% of the control).

Finally we tested whether blood digestion and protease activity were also inhibited when FU was given in the blood meal. Six-day-old females were fed 0.2% FU through membranes (4 ml. citrated sheep blood were mixed with 1 ml. of 0.85% NaCl containing 10 mg. FU). The results in Table V show that blood digestion was retarded in the treated females, and they also had less protease than the controls. The treated females did not lay any eggs, and their ovaries did not develop beyond stage 4.

Blood digestion in the controls fed diluted citrated sheep blood was slower than in the rat-fed untreated females. The amount of protease one day after membrane feeding was also somewhat lower than after feeding on a rat. Two days after membrane feeding 67% of the controls still had blood-filled midguts, and there was considerable protease activity. All controls laid eggs (an average of 69.5 eggs laid per female) but one day later than after feeding on a rat. The problem

TABLE V

Effect of feeding FU in the blood meal on blood digestion, midgut protease activity, and ovarian development. (FU in sheep blood fed through membranes)

Day after blood meal	% with full midguts		Average proteolytic activity*		Average stage of ovaries**	
	Controls	0.2% FU	Controls	0.2% FU	Controls	0.2% FU
1	100	100	0.61	0.33	5.3	2.7
2	67	92	0.47	0.36	6.8	2.8
3	0	8	—	—	8.0	3.1

* Mg. azoalbumin per three midguts, 12–20 blood-filled midguts in each test.
** Average includes all females, irrespective of the stage of blood digestion.

whether the delay in blood digestion and maturation of eggs was due to the dilution of the blood by 0.85% NaCl (4:1), the presence of citrate, or the fact that sheep blood rather than rat blood was fed, was not investigated.

One week after membrane feeding a second blood meal was given on a rat. Maturation of eggs and blood digestion in the controls were normal; all had stage 8 ovaries and had completed blood digestion 48 hours after feeding. Blood digestion in the treated group was retarded (50% digestion time—61 hours). The recovery of the ability to lay eggs in the treated group seemed to be an all-or-none process. Some females (36%) laid an almost normal number (average 75) of viable eggs, though later than the controls. In the females that did not recover their ability to mature eggs the follicles were small (about the size typical of stage 3–4 ovaries) and of irregular shape.

The relation between protease activity and ovarian development

In normal females the stage of ovarian development at various intervals after feeding on a rat was closely related to the stage of blood digestion (as estimated

by the color of the blood mass). When most ovaries have left the resting stage (stage 1) and are in stage 2, blood digestion has just begun; the blood mass is still bright red except for a thin brownish layer around the periphery. By the time the ovaries have reached stage 4–5 only the center retains its red color. In females with stage 6 ovaries the whole blood mass is brown. After that elimination begins.

In FU-treated females blood digestion and ovarian development were retarded to the same degree. Individuals with undigested blood had undeveloped ovaries; in the females where blood digestion had advanced further, the ovaries were also more developed. This holds true for all midguts that were still distended with the blood meal, irrespective of the day of dissection. Females treated with higher con-

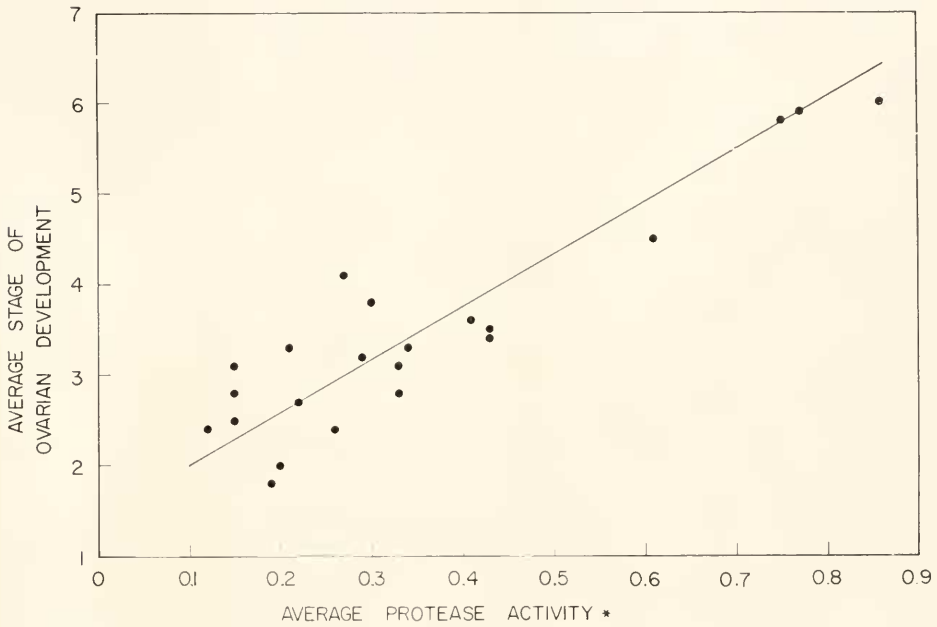

FIGURE 3. Correlation between protease activity and the stage of ovarian development. * Mg. azoalbumin digested by three blood-filled midguts in 30 minutes at 39° C.

centrations of FU attained a given stage of digestion and ovarian development later than those treated with lower concentrations of FU, or untreated ones.

The stage of the ovaries of each female whose midgut was taken for the protease assay in Table III was noted (only blood-filled midguts were tested, *i.e.*, before their peak protease activity or near it). In Figure 3 the average stage of the ovaries was plotted against the average protease activity ($n = 22$; 18 experiments from Table III in addition to four experiments with 0%, 0.0125%, 0.025% and 0.05% FU). The correlation coefficient is 0.872; the true correlation at the 95% level of probability is not less than 0.75 (Ezekiel and Fox, 1959). The amount of proteolytic activity is thus closely related to the stage of the ovaries in untreated and treated mosquitoes.

The effect of protease inhibition in vivo on ovarian development

In order to test the effect of protein digestion on the development of the ovaries, we tested the effect of crystalline soy bean trypsin inhibitor (CSBTI) *in vivo*. (Preliminary experiments have shown that CSBTI completely inhibits mosquito protease *in vitro*.) CSBTI was fed to 6-day-old mosquitoes in citrated sheep blood, 625 μg. per ml. Each engorged female thus obtained 1.25 μg. inhibitor in a 2-mg. blood meal.

One day after feeding there was no sign of blood digestion in the treated females; *i.e.*, the color of the blood mass was bright red. Eighty per cent of the ovaries were still in the resting stage (stage 1), only 20% reached stage 2. (The average stage of the control ovaries was 4.4.) Two days after feeding blood digestion began in some of the treated females. The stage of the ovarian development also varied greatly among individual females: 20% were still in the resting stage, the remainder in stages 3–6. The average stage of ovarian development in the treated group was 3.6 as compared with 6.0 in the controls. Three days after feeding the controls had finished blood digestion, and their ovaries contained mature eggs. The delay in blood digestion and ovarian development was still evident in the treated groups three days after feeding. Fifty per cent of the midguts contained blood, 25% of the ovaries were in stage 6, 25% in stage 7, and the remainder contained mature eggs and had empty midguts. Four days after feeding, 7% of the treated females still contained blood. The treated females oviposited later than the controls; the average number of eggs was 37 (48 in the controls). All eggs hatched normally.

The amount of protease in the treated females was 0.17 mg. azoalbumin per three midguts one day after feeding. This probably represents the protease contained in the midgut cells; the protease which reached the lumen was inactivated *in vivo* by CSBTI, since no blood digestion was observed (by the color of the blood mass). Protease activity in the treated group increased on the next day (0.56 mg.). Three days after feeding (when 50% of the treated females still contained blood) the average protease activity in the blood-filled midguts was 0.62 mg.

In untreated females the amount of protease was 0.43 mg. one day after feeding. Two days after feeding all controls contained blood, and the amount of protease was 0.46 mg. (Possibly the two points of protease measurement did not include the peak activity. Ovarian development and blood digestion in the membrane-fed controls in this experiment was more delayed than in the experiment reported in Table V.)

The results of this experiment show that it is possible to inhibit ovarian development by inactivating the midgut protease. It is possible that with higher concentrations of CSBTI, ovarian development could be completely arrested.

Discussion

The results of the present study show that FU inhibits midgut protease production and ovarian development in *Aedes aegypti* females. A similar correlation between the inhibition of ovarian development and midgut protease has been found by Thomsen and Møller (1963) after the removal of the medial neurosecretory

cells in *Calliphora*. The trypsin activity in the cockroach, *Nauphoeta cinerea*, is also related to oocyte development (Rao and Fisk, 1965).

The effect of FU in mosquitoes can be interpreted in two ways: (a) The effect of FU on the ovaries might be indirect, due to the deficiency in protease; (b) It is also possible that FU exerts a direct effect on the synthesis of yolk proteins, in addition to its indirect effect.

The results of this study do not enable us to decide between the two possibilities.

Our experiments on the feeding of crystalline soy bean trypsin inhibitor have shown that the inactivation of protease *in vivo* suffices to inhibit ovarian development. Other experiments in this laboratory (Weissman-Strum, unpublished results) have shown that the ovaries of *A. aegypti* females fed insufficient amounts of protein develop asynchronously. Such asynchronous ovaries were also observed in FU-treated females. The toxicity of FU in mammals is mainly due to its effect on the alimentary tract (Muggia *et al.*, 1963). If the main damage by FU in mosquitoes is also located in the midgut cells, the production of intestinal protease (and possibly also the absorption of the nutrients) would be impaired. The effect of FU would thus be akin to that of protein starvation.

Studies on the mode of action of FU in microorganisms and experimental neoplasms have shown (see review by Brockman and Anderson, 1963) that in order to become active FU must first be metabolized to fluorouridylic acid and subsequently incorporated into RNA (or be converted to 5-fluorodeoxyuridylic acid which blocks the synthesis of thymidylic acid). It is assumed that FU acts on the rapidly dividing cells in the reproductive system in insects by a similar mechanism (Bøřkovec, 1962). Our results show that the effect of FU in mosquitoes is not confined to the ovaries.

Incorporation of FU into RNA is known to produce various abnormalities in protein synthesis (Brockman and Anderson, 1963). Incorporation of FU into RNA results in the synthesis of altered enzymes which are enzymatically inactive (Gros and Naono, 1961). Vitellogenesis entails many metabolic processes, involving the synthesis of specific proteins in all parts of the insect body (Telfer, 1965). In *A. acgypti* the yolk proteins are not synthesized in the oocyte itself, but taken up from the haemolymph by micropinocytosis (Roth and Porter, 1964). This uptake is initiated by a blood meal; it is possible that the synthesis of the yolk proteins is similarly triggered. Serological gel-diffusion tests have shown that several proteins, not present in unfed females (and different from the egg proteins), appear after a blood meal in *A. acgypti* (Edman, 1964). These proteins might represent the enzymatic systems which are initiated by a blood meal. Interference by FU with any of these processes which involve the synthesis of specific enzymes, would inhibit the growth of oocytes. Midgut protease might be only one of several enzyme systems which are inhibited by FU, and whose direct result would be the inhibition of egg maturation.

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Summary

1. Oviposition in *Aedes aegypti* females is inhibited by 5-fluorouracil (FU); 0.01% FU fed in the blood meal causes about 99% inhibition of oviposition. The inhibition of oviposition by FU is temporary; some of the females which laid no eggs after their first blood meal recover their ability to oviposit after a second blood meal. The eggs laid by the FU-treated females hatch normally.

2. Oviposition in mosquitoes is also inhibited by feeding FU in sugar solution before the blood meal; 0.05% causes about 95% inhibition of oviposition when given in sugar solution for two days. This effect is also temporary. The eggs laid by females treated with FU in sugar solution hatch normally even when both sexes have been treated.

3. FU slows down the growth of oocytes; the treated females oviposit later than the controls. The rate of oocyte growth and the percentage of females developing mature eggs is proportional to the amount of FU.

4. Blood digestion is retarded by FU-treatment. The delay in blood digestion is already evident one day after feeeding by the color of the blood mass. The period of blood digestion in females treated with 0.05% FU in sugar solution is more than twice that in the controls.

5. Midgut proteolytic activity is inhibited in FU-treated females. The extent of inhibition is proportional to the amount of FU and the time interval between FU-treatment and the blood meal.

6. FU does not inactivate protease *in vitro*.

7. A close correlation between the stage of ovarian development and the amount of midgut protease has been found.

8. Soy bean trypsin inhibitor, which inactivates mosquito protease *in vitro*, also retarded the maturation of eggs *in vivo*.

9. The effect of FU on oocyte maturation in mosquitoes could be an indirect effect (oocyte growth is inhibited because of a lack of midgut protease) or a direct effect of FU on the synthesis of specific proteins in all parts of the insect body.

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